Original Article

Pramlintide: An Amylin Analogue Protects Endothelial Cells against Oxidative Stress through Regulating Oxidative Markers and NF-κb Expression

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Abstract

Background: Oxidative stress has a prominent role in the pathogenesis of diabetes complications. Pramlintide is an injectional amylin analogue used for the treatment of type 1 and type 2 diabetic patients. The present investigation evaluated the effect of pramlintide against oxidative damage induced by hydrogen peroxide (H_2O_2) in human umbilical vein endothelial cells (HUVECs). **Methods:** Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. Hydroperoxides level, ferric reducing antioxidant power (FRAP), and expression of transcription factor NF-κB were measured in HUVECs that pretreated with pramlintide and, then exposed to H_2O_2. **Results:** Pramlintide significantly decreased the cytotoxicity caused by H_2O_2 at the concentrations of 5 and 10 μg/mL. Pretreatment of HUVECs with pramlintide reduced hydroperoxides and increased FRAP value in intra- and extra-cellular mediums at different concentration ranges compared with H_2O_2 stimulated cells. Pramlintide (10 μg/mL) remarkably ameliorated the expression of NF-κB gene after 1, 3 and 24 h exposure to H_2O_2. **Conclusions:** Findings of the current investigation displayed that pramlintide may act as a protective against oxidative conditions in endothelial cells through modulation of oxidative markers and transcription factor NF-κB.

Keywords: *Diabetes complications, human umbilical vein endothelial cells, NF-kappa B, oxidative stress*

Introduction

The growing incidence of diabetes is one of the most serious human problems in the 21st century. By 2030, about 578 million and by 2045, near 700 million people will develop diabetes worldwide, according to the report of International Diabetes Federation Diabetes Atlas.[1] Micro-vascular and macro-vascular problems are the prominent causes of morbidity and mortality in diabetic patients.[2] The role of endothelial dysfunction and its strong relationship with oxidative stress has been identified in the pathophysiology of various cardiovascular disorders.[3] Hyperglycemia leads to the high formation of reactive oxygen species (ROS) and weakness in the antioxidant protection system and therefore to the various oxidative injuries in the vasculature.[4] Recent evidence has shown that high levels of glucose stimulate hydrogen peroxide (H_2O_2) production and activates the nuclear factor-kappa B (NF-κB) in human endothelial cells.[5] NF-κB as a transcription factor is critically participated in inflammatory and oxidative stress signaling pathways through prompting the expression of pro-apoptotic genes and pro-inflammatory mediators and subsequently in the vascular dysfunction in diabetes.[6]

Pramlintide is a synthetic amyloid analogue and an injectable antidiabetic agent which has been approved for use in type 1 and type 2 diabetic patients who do not reach the desired glucose level with taking insulin. It modulates the postprandial glucose levels with similar mechanisms to amylin.[7] Amylin which is secreted from pancreatic beta cells in response to high post-meal blood sugar along with insulin, reduces the level of blood glucose by slowing gastric emptying, inhibiting secretions of glucagon and some gastrointestinal hormones, and reducing appetite and food intake.[8] Recently, some limited reports have described the antioxidant activities for pramlintide. This drug has been able to reduce the

How to cite this article: Safaeian L, Shafiee F, Naderi M. Pramlintide: an amylin analogue protects endothelial cells against oxidative stress through regulating oxidative markers and *NF-κb expression*. Int J Prev Med 2022;13:20.

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Access this article online

Website: www.ijpvmjournal.net/www.ijpm.ir

DOI: 10.4103/ijpvm.IJPVM_425_20

Quick Response Code:

oxidative stress markers, including oxidized-low density lipoprotein (LDL) and nitrotyrosine, and to preserve total radical trapping parameter in diabetic patients and also to reduce the expression of a lipid peroxidation product in Alzheimer's disease model.[9-11]

The present experiment was conducted to better understand the mechanisms underlying in antioxidant effects of pramlintide through evaluation of alteration in the expression of transcription factor NF-κB and oxidative stress markers including hydroperoxides level and total antioxidant capacity under condition of oxidative damage caused by H_2O_2 in human umbilical vein endothelial cells (HUVECs).

Methods

Cell culture

HUVECs were obtained from National Cell bank (Pasteur Institute, Tehran, Iran). The cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotics (penicillin-streptomycin) in an incubator under condition of 95% humidified air with 5% CO_2 at 37°C.

Cell viability assay

3-4,5-Dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) kit (Bioidea Co., Tehran, Iran) was used for determining the effect of pramlintide (AstraZeneca *Co*., Cambridge, UK) on viability of HUVECs during normal and oxidative stress conditions.[12] Briefly, a density of 1×10^4 cells were seeded per each well of 96-well plate and incubated with pramlintide (1-10 μg/mL) or vitamin C (Vit C; 10 μg/mL, positive control) for 24 h. After adding MTT reagent and incubation for 3 h, and then mixing with DMSO, absorbance was read using a microplate reader/spectrophotometer (BioTek Instruments, PowerWave XS, Wincoski, USA) at 570 nm. For evaluation of the cytoprotective activity of pramlintide, 24 h-pretreated cells were washed out with a buffer and then exposed to H_2O_2 (0.5 mM) for 2 h. The rest of the test was done as above. All experiments were performed three independent times, each in triplicate. The viability of treated HUVECs was assessed as percentage of untreated control group.

Hydroperoxides content assay

The content of hydroperoxides was assessed using ferrous ion oxidation by xylenol orange (FOX-1) kit (Hakiman Shargh Research Co., Isfahan, Iran) in intra- and extra-cellular mediums of treated cells. In brief, HUVECs were incubated with pramlintide (1-10 μg/mL) or Vit C (10 μg/mL) for 24 h and then with H_2O_2 for 2 h. After that, 190 μL of FOX-1 reagent was added to 10 μL of supernatant or cell lysates from each well and incubated for 30 min at 40°C. Finally, absorbance was read at 540 nm by a microplate reader/spectrophotometer. The contents of hydroperoxides in tested samples were calculated as H_2O_2 equivalents using a standard curve of H_2O_2.[13]

FRAP assay

Total antioxidant capacity was measured using a ferric reducing antioxidant power (FRAP) kit (Hakiman Shargh Research Co., Isfahan, Iran) in intra- and extra-cellular fluids. FRAP reagent (200 μL) was added to 10 μL of supernatant or cell lysates of treated cells. After incubation at 40°C for 40 min, absorbance was read at 570 nm by a microplate reader. FRAP values of tested samples were calculated using a standard curve of $FeSO_4$ and stated as μM of FeII equivalents.[14]

Gene expression assay by real-time quantitative PCR

Total RNA was extracted from treated and untreated HUVECs after 1, 3, and 24 h incubation using BIOFACT™ Total RNA Prep kit (BioFact Ltd., Daejeon, Korea) according to the manufacturer's instruction. Measurement of concentration of RNA samples was performed using a NanoDrop system spectrophotometer at 260/280 nm.

Total RNA was reverse transcribed to cDNA with oligo-dt primers using Yekta Tajhiz Azma kit (Tehran, Iran) and then in order to quantitatively examination of NF-κB gene expression, quantitative real-time RT-PCR was performed on StepOne™ Real-Time polymerase chain reaction (PCR) System (USA). Reaction mixture (10 μL) containing 1 μL of cDNA template, 1 μL of mixture of forward and reveres primers and Quantitect SYBR Green master mix (Qiagen, Hilden, Germany) amplified based on SYBR Green method. Direct detection of PCR products was monitored by measuring the fluorescence produced due to SYBR Green dye binding to dsDNA after every cycle. Each cycle of amplification was as follow: denaturation at 95°C for 15 min and 45 cycles at 95°C for 20 s, 60°C for 30 s and finally, 72°C for 30 s. The primers used in this study were designed by vector NTI version 11 and synthesized by Genfanavaran Co. (Tehran, Iran) and quantification of NF-κB gene expression was normalized to the endogenous glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene using the $2^{-\Delta\Delta Ct}$ method. The following primer sequences were used: NF-κB forward 5'-GCA TGC CAA TGC CCT TTT CG-3' and NF-κB reverse 5'-GCA CAG CAG TGA GAT GGC G-3', GAPDH forward 5'- CTC CCG CTT CGC TCT CTG-3' and GAPDH reverse 5'-TCC GTT GAC TCC GAC CTT C-3'.

Statistical analysis

The results were indicated as mean ± standard error of mean. All statistical tests were performed by SPSS software (version 25.0) by One-way analysis of variance and Tukey post-hoc test. Findings were considered significantly different when $P < 0.05$.

Results

Effect of pramlintide on HUVECs viability

Viability of HUVECs was measured by MTT assay after 24 h exposure to pramlintide. Treatment with pramlintide at the concentration range of 1-10 μg/mL caused no inhibitory effect on HUVECs proliferation [Figure 1a].

As shown in Figure 1b, exposure to H_2O_2 for 2 h resulted in a significant decrease in HUVECs viability ($P < 0.001$). Pretreatment of cells with pramlintide at 5 and 10 μg/mL significantly prevented the cytotoxicity induced by H_2O_2 ($P < 0.01$ and $P < 0.001$, respectively).

Effect of pramlintide on hydroperoxides content

Hydroperoxides level was notably elevated in intra- and extra-cellular fluids of HUVECs during oxidative stress caused by H_2O_2 ($P < 0.001$). Incubation of cells with pramlintide resulted in a significant reduction in the hydroperoxides level in intra- and extra-cellular mediums at all concentrations (1–10 μg/mL) compared with H_2O_2 stimulated cells ($P < 0.001$) [Figure 2].

Effect of pramlintide on FRAP value

A significant reduction in FRAP value was observed in intra- and extra-cellular mediums of HUVECs after exposure to H_2O_2, ($P < 0.01$). Pretreatment with pramlintide meaningfully increased FRAP value in extracellular fluids at all concentrations. However, it was elevated FRAP value in intracellular fluids at the concentrations of 5 and 10 μg/ml compared with H_2O_2 stimulated cells [Figure 3].

Effect of pramlintide on *NF-κB* gene expression

A significantly higher rates of NF-κB expression were observed after 1, 3 and 24 h incubation times in H_2O_2 stimulated cells ($P < 0.001$). Pramlintide (10 μg/mL) notably decreased NF-κB expression after 1 h ($P < 0.05$), 3 h ($P < 0.01$) and 24 h ($P < 0.001$) exposure to H_2O_2 in HUVECs [Figure 4].

Discussion

In this study, *in vitro* evaluation of pramlintide as an amylin analogue showed protective and antioxidant activities through elevation in cellular viability and in FRA*P* value, and reduction in hydroperoxides concentration and NF-κB gene expression in HUVECs under condition of oxidative toxicity caused by H_2O_2.

Amylin is a member of calcitonin gene peptide superfamily. This superfamily consist of calcitonin gene-related peptide, calcitonin, amylin, and adrenomedullin which possess some similar physiological functions because of likeness in their structures and receptors.[15] Their receptors are spread in numerous tissues, like neuronal, cardiac, and vascular tissues.[16] In endothelial cells, there are evidences that these members inhibit apoptosis, stimulate angiogenesis. and mediate endothelium-dependent vasodilation.[17,18]

In our study, pramlintide at concentration range of 1–10 μg/mL showed no inhibitory effect on HUVECs viability during normal condition. It considerably prohibited the cytotoxicity caused by H_2O_2 at the concentrations of 5 and 10 μg/mL. Wu *et al*.[19] evaluated the effect of pramlintide on viability of human nucleus pulposus cells during normoxia and hypoxia at concentration range of 250–500 nM (almost 1-2 μg/mL) and showed neuroprotective effect of pramlintide through preventing mitochondrial-dependent apoptosis.

Moreover, our findings showed declining in hydroperoxides concentrations and increasing in FRAP value in intra- and extra-cellular mediums after pretreatment with pramlintide. However, there was more prominent effect by pramlintide on reducing the intra-cellular hydroperoxides level and elevating the extra-cellular FRAP value. Pramlintide also ameliorated the expression of NF-κB gene.

There are only a few reports about the helpful effects of pramlintide on oxidation indicators. In the randomized, single-blind studies on 18 type 1 and 19 type 2 diabetic patients conducted by Ceriello *et al*.,[9,10] pramlintide showed antioxidant effects through decreasing oxidized-LDL and nitrotyrosine as a reactive nitrogen specie. They also described improving effect for pramlintide through preserving total radical trapping parameter as a total amount of plasma antioxidant capacity during hyperglycemic oxidative status.[9,10] In the study of Adler *et al*., pramlintide reduced expression of cyclooxygenase 2 as an inflammatory indicator and expression of 4-hydroxynonenal as a lipid peroxidation product during oxidative stress in the brain of a mouse model of Alzheimer's disease.[11]

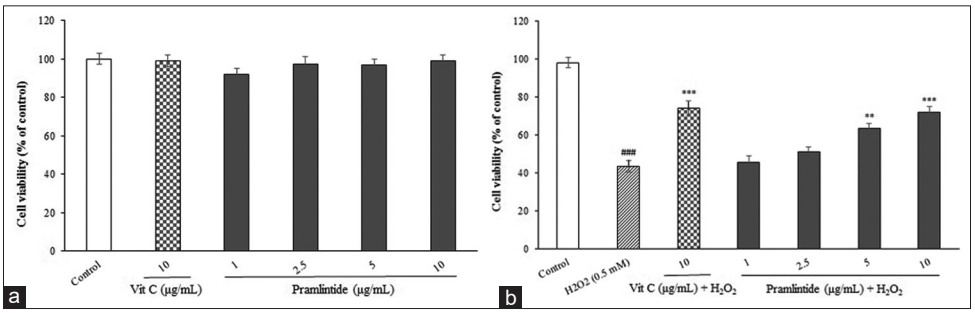

Figure 1: Effect of pramlintide on HUVECs viability during normal condition (a) and H_2O_2-induced oxidative stress (b) determined by MTT assay. Values are means ± SEM. ###*P* < 0.001 versus control (untreated cells), ***P* < 0.01 and ****P* < 0.001 versus H_2O_2 stimulated cells

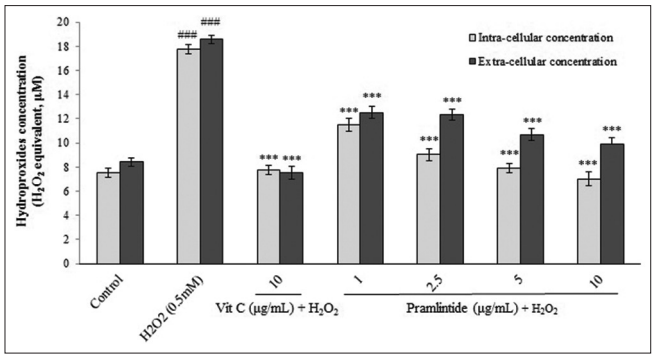

Figure 2: Effect of pramlintide on intra- and extra-cellular hydroperoxides concentration during H_2O_2-induced oxidative stress in HUVECs determined by FOX-1 method. Values are means ± SEM. ###*P* < 0.001 versus control (untreated cells), and ****P* < 0.001 versus H_2O_2 stimulated cells

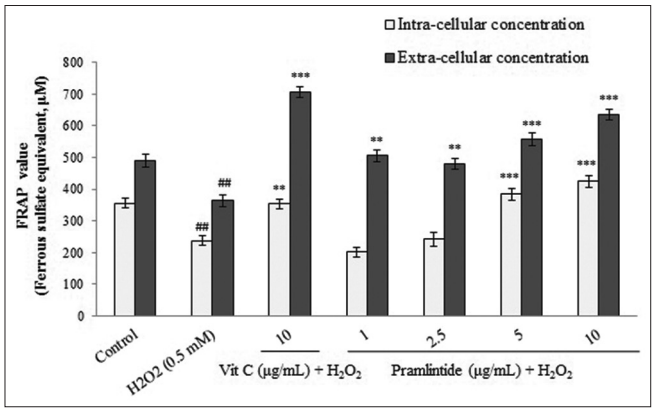

Figure 3: Effect of pramlintide on intra- and extra-cellular FRA*P* value during H_2O_2-induced oxidative stress in HUVECs. Values are means ± SEM. ##*P* < 0.01 versus control (untreated cells), **P* < 0.01 and ***P* < 0.001 versus H_2O_2 stimulated cells

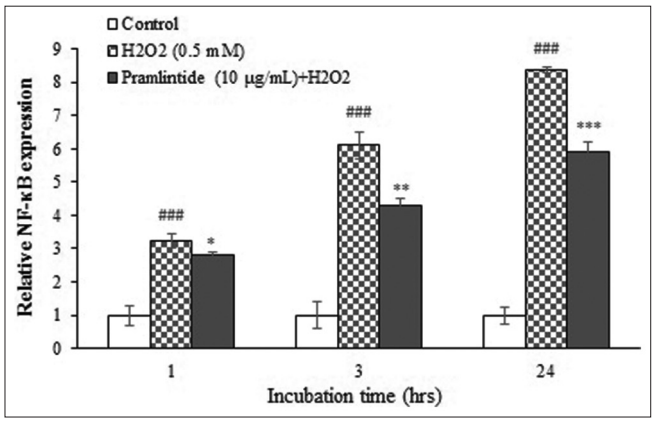

Figure 4: Effect of pramlintide on NF-κB gene expression during H_2O_2-induced oxidative stress in HUVECs determined by real-time quantitative PCR. Values are means ± SEM. ###*P* < 0.001 versus control (untreated cells), and **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 versus H_2O_2 stimulated cells

Furthermore, some protective activities against oxidative stress have been established for other members of calcitonin gene peptide superfamily. It is well known that adrenomedullin act as a vascular protectant antioxidant by inhibiting intracellular ROS production through a cAMP-dependent pathway during oxidative condition induced by angiotensin in endothelial cells.[20] Adrenomedullin has also resulted in a declining in NF-kB activation during inflammatory status in hepatic cells.[21] CGRP as another member has shown antiapoptotic and antioxidant activities against high-glucose condition in dorsal root ganglion neurons by reducing malondialdehyde and ROS amounts through initiation of the phosphatidylinositol 3-kinase (PI3K)/AKT signaling and subsequently elevation of nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 expression.[22]

Regarding the role of NF-κB in inflammation and oxidative stress signaling pathways in endothelial cells and important correlation between oxidative stress and endothelial dysfunction,[3,5] declining NF-κB and ROS production, and enhancement of antioxidant power by pramlintide may improve the endothelial function and subsequently prevent various micro- and macro-vascular problems in type 1 and type 2 diabetic patients.

Conclusions

In conclusion, findings of the current study indicated that pramlintide may act as a protective against oxidative conditions in endothelial cells through modulation of oxidative markers and transcription factor NF-κB. Regarding the role of oxidative stress and NF-κB in the pathogenesis of diabetes and its problems, additional studies are suggested to confirm the potential role of pramlintide for treatment of diabetic vasculopathy.

Financial support and sponsorship

This study was financially supported by Vice-Chancellery for Research and Technology of Isfahan University of Medical Sciences (research projects No. 397614).

Conflicts of interest

There are no conflicts of interest.

Received: 26 Jul 20 **Accepted:** 26 Jun 21
Published: 08 Feb 22

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